Production of Riboflavin by Fermentation

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The term "vitamin" has reached everyday usage, yet it cannot be defined readily. Vitamins are organic compounds and are requisite to the normal growth of man and animals. They are not generally produced within the animal body; they must be derived from the food consumed. Vitamins do not become a part of body cells, but are essential for the metabolic processes conducted by the cells.

One of the important vitamins is riboflavin, sometimes referred to as lactoflavin, vitamin B_2, or vitamin G. It belongs to the group that is soluble in water. Riboflavin deficiencies, like deficiencies of other vitamins, are marked by poor rates of growth and inefficient utilization of food by animals. Severe or complete deficiencies can lead to death.

Riboflavin is rather widely distributed in food and feedstuffs, but in amounts generally inadequate for high animal efficiency. High-potency sources of the vitamin have been needed for enriching such foods and feeds. Today riboflavin is produced in quantity by chemical and fermentation processes. Small but nutritionally substantial amounts are incorporated in most bread flours and breakfast foods, in some pharmaceuticals, and in nearly all poultry and hog feeds.

The vitamin activity of riboflavin is well recognized today, but some 50 years passed before its vital importance in animal nutrition was proved. Nearly 70 years ago, A. W. Blythe, an English chemist who was studying the composition of milk, directed attention to a water-soluble yellowish pigment, which he termed "lactochrome." Other chemists succeeded in concentrating the pigment and in 1925 recorded some of its more obvious characteristics, but they considered it solely as one of the minor constituents of milk. That was at the beginning of the era when biochemists intensified their studies of the relationship between food composition and the growth and well-being of animals. The reports of Christian Eijkman (1897) and others had demonstrated already that a disease, beriberi, common in the Dutch East Indies, was of nutritional origin. Casmir Funk, an American, also interested in nutritional diseases, a few years later (1911) coined the term "vitamine" for the trace chemicals in foods that are responsible for promoting normal well-being in animals.

Workers soon found that two classes of vitamins existed, one class being essentially fat-soluble, which they first termed "vitamin A", and another they called "water-soluble B." The multiple nature of each class became obvious as research progressed. By 1929 two of the water-soluble group, vitamin B_1 (thiamine) and vitamin B_2 or G (riboflavin), were distinguished on the basis of greater heat stability of vitamin B_2.

The identification of any new chemical of therapeutic importance involves five major steps. Its existence is recognized by appropriate biological tests. Concentrates and later the pure compound are prepared by isolation from natural materials. The chemical structure of the compound derived from natural substances is determined. The compound is reproduced by chemical synthesis. The natural and man-made

Riboflavin requirements established by the National Research Council, 1946

Livestock	Riboflavin requirement Milligrams per pound of feed
Chicks:	
Starting (0–8 weeks)............	1.6
Growing (8–18 weeks)..........	.9
Hens:	
Laying.......................	.9
Breeding.....................	1.3

	Milligrams a day
Swine (growing-fattening pigs):	
50 pounds...................	2.1
100 pounds..................	3.8
150 pounds..................	5.0
200 pounds..................	5.7
250 pounds..................	5.7

	Milligrams per pound of food	
Humans	Male	Female
Children:		
Under 1 year..............	0.6	0.6
1–3 years.................	.9	.9
4–6 years.................	1.2	1.2
7–9 years.................	1.5	1.5
10–12 years...............	1.8	1.8
13–15 years...............	2.4	2.0
16–20 years...............	3.0	1.8
Adults:		
Sedentary.................	2.2	1.8
Moderately active.........	2.7	2.2
Very active...............	3.3	2.7
Pregnancy, latter half......	......	2.5
Lactation.................	......	3.0

compounds are tested biologically to prove their identity.

The identification of riboflavin as a vitamin stemmed from several scientific fields. In 1932 Otto Warburg and Walter Christian, in Germany, isolated a new oxidation enzyme from yeast. Water solutions of the enzyme were yellow, but they exhibited a characteristic greenish fluorescence when viewed in ultraviolet light. By brilliant work, the investigators succeeded in separating their enzyme into two parts, a protein and a yellow pigment.

Animal nutritionists, notably Lela Booher, then at Columbia University, had suspected that the yellow pigment in whey might be responsible for the prevention of the characteristic vitamin B_2 deficiencies in rats. Phillipp Ellinger and Walter Koschara suggested it might be related to the yellow pigment described by Warburg and Christian. In 1935 two groups of European chemists, directed respectively by Richard Kuhn in Germany and Paul Karrer in Switzerland, succeeded in synthesizing the yellow pigment in the laboratory. Animal-feeding tests proved that the laboratory product was the same as that obtained from yeast.

RIBOFLAVIN is the common name for 6,7-dimethyl-9-(d-1'-ribityl)-isoalloxazine. The molecule has the following structure:
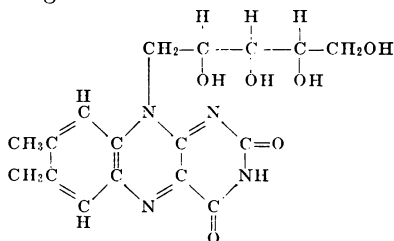
Pure riboflavin has needle-shaped, practically odorless, orange-yellow crystals, which begin to darken at about 240° C. (464° F.) and completely decompose at about 280° C. (536° F.). Water solutions show a characteristic yellowish-green fluorescence.

Riboflavin is slightly soluble in water (12 milligrams in 100 milliliters at 27.5° C.; 19 milligrams at 40° C.) and in several organic solvents. It is very soluble in alkali. Solutions are relatively stable to acid, but riboflavin is readily destroyed by alkali and light. In neutral water solutions, the compound exhibits a characteristic light absorption spectrum, with maxima at 445, 365, 265, and 220 millimicrons.

Riboflavin occurs in nature in free and "bound" forms. In urine it is entirely free, in milk principally free, but

in plant and animal tissues it is largely combined with proteins, which are called flavoproteins. These are yellow enzymes in which riboflavin mono-nucleotide (riboflavin phosphate) or riboflavin dinucleotide (riboflavin phosphate and adenosin phosphate) is combined with specific proteins. Several such oxidizing enzymes are known, each with specific functions in tissue metabolism.

The water-soluble B-complex vitamins appear to serve as biologically active portions of enzymes. Enzymes are responsible for releasing energy from food; they also effect the chemical reactions associated with the synthesis of new body tissue and the repair of damaged tissue. Life itself is the result of a very complex series of enzyme reactions, for each of which nature has provided appropriate regulatory means. A vast amount of research has thrown light on this complex picture, yet much remains to be done before man can fully understand the wonders of life and the processes that sustain it.

DISEASES OF ANIMALS AND PLANTS can be classified into two general groups—those caused by the invasion of disease-producing micro-organisms and viruses, and those caused by inadequate or unbalanced nutrition, which impairs the functioning of tissue cells. The vitamins are especially effective in treating diseases of the second group.

In animals, riboflavin deficiencies are shown in many ways. The degree of deficiency may vary considerably and, of course, the appearance of the specific symptoms will vary accordingly. Retarded growth is common to all classes of animals. "Curled-toe paralysis" is a symptom in chicks, and poor hatchability is a result of such deficiency. Dermatitis and eye cataracts are frequent among rats. Dogs, in acute deficiency, exhibit a characteristic collapse; they may die within 6 to 8 weeks. Chronic deficiencies lead to nervous abnormalities.

In man, abnormal conditions in the eyes generally appear before other symptoms. As the deficiency progresses, symptoms include inflammation of the lips and tongue, fissures at the corners of the mouth, dermatitis, and other less specific conditions.

VITAMIN REQUIREMENTS depend on the age of the animal and the physiological functions it performs. Non-ruminant animals, such as man, swine, and poultry, require preformed B-complex vitamins in their food. Ruminants, such as sheep and cattle, do not. There is some evidence that B-complex vitamins should be supplied to young calves until the rumen functions. The micro-organisms in the rumen then produced sufficient B-vitamins for the animal.

Committees of the National Research Council have recommended minimum amounts of dietary riboflavin for humans, poultry, and swine.

From all these data, we learned that chemical structure of riboflavin and the advent of suitable analytical methods, foods and feeds were analyzed for their content of the vitamin. Simultaneously, animal nutritionists engaged in studies that established quantitatively the requirements by various animals.

From all these data, we learned that the usual animal rations, particularly those composed largely of cereal grains, contained inadequate amounts of riboflavin for the best rate of growth. Milk products, such as whey or skim milk, supplied the additional riboflavin needed, but milk products are expensive. To meet the need for additional sources of the vitamin, organic chemists began producing riboflavin by chemical means. The synthetic vitamin appeared on the market in 1938. Since then, it has been produced in large quantities at progressively lower cost.

Used to fortify feeds, however, riboflavin concentrates are just as efficient as the pure compound. The fermentation industry, which produces chemicals such as alcohol, butanol, and acetone from grain and molasses, has long

been confronted with the problem of the disposal of its fermentation residues. The residues were found to be good sources of riboflavin and other vitamins. They contain the vitamins originally in the grain, in addition to those produced by the fermenting micro-organisms. By proper drying methods, the vitamins were concentrated into useful products. Progressive members of the industry immediately undertook to recover the residues. The byproducts from grain fermentations are now marketed as distillers' light grains, distillers' solubles, and distillers' dark grains, the latter being essentially a mixture of the first two products.

The need for acetone during the First World War led to the development of butanol-acetone fermentation, which continues as an important chemical process. Research workers frequently had noted that a yellow pigment became pronounced in the course of grain fermentations, even before riboflavin was known. Some workers suspected that the pigment was being extracted from corn by the solvents made by the fermenting organism, *Clostridium acetobutylicum*. However, by 1940, research confirmed that the pigment was riboflavin synthesized by this bacterium as it produced the neutral solvents, butanol and acetone.

Methods of improving riboflavin yields without impairing solvent yields were soon devised. The byproduct residues of this fermentation became an important source of riboflavin concentrates for feed fortification. The fermentation is now applied also to whey and other milk byproducts to obtain rich concentrates for poultry and livestock.

In 1935, Alexander Guilliermond, a French mycologist, observed that a yeastlike organism, *Eremothecium ashbyii*, originally isolated as a pathogen for cotton plants in the Belgian Congo, produced a yellow pigment in laboratory cultivation. In fact, microscopic examination revealed crystals of the pigment within the threadlike cells. Later research, particularly in the United States, proved that the organism could produce the vitamin in such amounts that riboflavin was the sole useful fermentation product.

Other yeasts, belonging to the *Candida* genus, produce significant amounts of riboflavin, but they are not applicable to commercial exploitation because of their extremely low tolerance for iron.

Guilliermond and his colleagues, when studying *Eremothecium ashbyii*, reported that a related species, *Ashbya gossypii*, produced only traces of riboflavin. In 1943 L. J. Wickerham, zymologist at the Northern Regional Research Laboratory, obtained a culture of *Ashbya gossypii* from W. J. Robbins, director of the New York Botanical Garden. After extended growth, the culture acquired a greenish-yellow cast. Suspicions that this pigment was riboflavin were confirmed. Using microbiological isolation procedures, Dr. Wickerham obtained a strain of the microorganism capable of producing the vitamin rapidly and abundantly under laboratory conditions. To promote industrial interest in this new fermentation process, an inexpensive substrate and conditions applicable to commercial practice have been developed.

IN THE FERMENTATION production of riboflavin by *Ashbya gossypii*, the culture medium, comprising glucose (corn sugar), corn steep liquor (byproduct of corn wet milling), and animal stick liquor (a packing-house byproduct of wet rendering), is prepared in a mixing tank. The medium is pumped at a controlled rate through a steam jet heater, where by injection of high-pressure steam the solution is almost instantaneously heated to 275° F. (135° C.). The hot solution circulates through insulated pipes to retain the high temperature for 5 minutes, then through additional pipes or coils surrounded by cold water to reduce the temperature to 82° to 86° F. (28° to 30° C.). Through steam-sterilized pipe

lines, the cooled solution is pumped to a sterile fermentation vessel. This is a closed tank equipped with a jacket or coils by which the tank contents may be maintained at a uniform temperature of 82° F. (28° C.). In the bottom of the tank are fine-porosity stones or perforated coils through which sterile air is supplied. A mechanical agitator assists in providing adequate air distribution.

After the sterile culture medium is transferred to the tank, a small volume of a day-old culture of *Ashbya gossypii* is added, and sterile air is introduced through the air distribution system at a rate of one-fourth to one-half volume per volume of medium per minute. By the fourth day, the maximum yield of riboflavin has been obtained, and the culture medium has acquired a beautiful, intense yellow color.

Two types of products can be produced. A potent riboflavin concentrate, ideally suited to enriching poultry and livestock feeds, can be had by evaporating the water from the fermented medium to prepare a sirup of about 30 percent solids. The sirup is converted to a dry powder by such conventional equipment as a drum or spray drier. The drum drier has a pair of cylindrical rolls, mounted horizontally, which are steam heated. The sirup is continuously fed to the valley between the rolls, and as the rolls rotate in opposite directions a thin film of sirup adheres to each. The water rapidly evaporates before a revolution is completed, and the dry material, scraped off by knives, is conveyed to bagging equipment. In the spray-drier method, the sirup is sprayed into a chamber through which heated air is passed; the air absorbs the water; and the dry riboflavin concentrate is mechanically removed to packaging equipment. Concentrates containing 25,000 micrograms of riboflavin per gram (11,350 milligrams per pound, or 2.5 percent riboflavin) are thereby produced.

Pure crystalline riboflavin may be recovered from the fermented solution. Synthetic riboflavin was marketed in 1938. The price was $7,945 a pound. Within a year the price was reduced to $3,642, and by 1944 it had dropped to $90 a pound. Despite substantially increased costs of manufacture during the war and afterward, improvements in the processes and the economies of mass production have cut the costs further. Riboflavin was priced at about $56 a pound in 1950. In 1943 more than 75,000 pounds of crystalline riboflavin was produced from all sources. Production figures are not available for the additional amounts in the form of high-potency concentrates that have been made by fermentation procedures.

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Illustrated on the next page are some steps in producing an insecticide. At the top are pyrethrum flowers, mentioned on page 767, which yield pyrethrins. The middle picture is a drawing of a model showing the structure of a molecule of the substance. Below is a device used to test the efficiency of an insecticide on insects.